The Combination Extract of Pare and Apples (APa) Reduces Risk of Atherosclerosis through Reduction of Interleukin 17 and Aggregate Focus of Liver Inflammation in High-Fat Diet Mice

Ni Made Linawati[1*], Ni Putu Sriwidyani[2], I Nyoman Wande[3], Arijana Kamasan[1], Sri Wiryawan[1], Dewi Ratnayanti[1], Wayan Sugiritama[1], Ika Wahyuniari[1]

[1] Department of Histology, Faculty of Medicine Udayana University, Indonesia.
[2]Department of Anatomy Pathology Faculty of Medicine Udayana University, Indonesia.
[3] Department of Clinical Pathology Faculty of Medicine Udayana University, Indonesia.

ABSTRACT

Atherosclerosis is a marked increase in IL-17 and fatty liver/ liver inflammation in a group with a high-fat diet. The study aimed to test the potential of the extract Apples Pare (APA) in reducing the risk of atherosclerosis by decreasing IL-17 and fatty liver in mice given a high-fat diet (HFD). This study used 35 mice as samples which were divided into 7 groups. K0 included mice with a normal diet; K1 mice were given HFD; P1 mice were given HFD and extract Pa; P2 mice were given the extract HFD and Ap; P3 mice were given HFD and extract APa; P4 mice were given HFD and Simvastatin; whereas P5 mice were given HFD, APa extract and Simvastatin. This study showed no fatty liver. However, there was a decrease of liver inflammation with the highest decline in a row in P5, P4, P2, P3, and final P1 than K1. This showed that the extract potentially would reduce the liver inflammation.

Key Words: *Extract APa, IL-17, Liver Inflammation.*

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INTRODUCTION

Obesity is a condition marked by the excessive accumulation of fat more that required for the normal body function. World Health Organization (WHO) has declared obesity as the biggest chronic health problem in adults. According to WHO, the prevalence of obesity worldwide more than doubled between 1980 and 2014. Obesity in European, American and Australian countries reached at epidemic levels, as well as in developing countries, and it has become a serious health problem. By 2014, there were over 1.9 billion adults (over 18 years) overweight. Of these, more than 600 million were obese. Overall, in 2014, about 13% of the adult world population (11% male and 15% female) are obese [1]. Increased obesity has shown an increase in coronary heart diseases which have been characterized by atherosclerotic plaque. The increased range of pro-inflammatory cytokines such as IL-6, TNF-α and IL-17 showed the increased formation of atherosclerotic plaque. Interleukin 17 secreted by Th17 cells play a role in atherosclerosis formation in obesity. The other adipocytes (adiponectin, leptin) get secreted by adipocytes, and there is also cytokine secretion from macrophage cells. IL-6, TNF-a, IL -1, MCP-1, and MIP-1a also showed their role in atherosclerosis [2, 3]. Pare and green apple extracts each have been shown to lower total cholesterol, LDL, trigriserida and lower blood sugar. The fruit contains bitter melon (*Momordica charantia*) containing saponins, flavonoids, polyphenols, alkaloids, triterpenoids, momordisins, glycosides cucurbitacin, charantin, butyric acid, palmitic acid, linoleic acid and steroids that have been shown to lower cholesterol, and

Corresponding author: Made Linawati
Address: Department of Histology Faculty of Medicine Udayana University, PB Sudirman Street Denpasar Bali.
E-mail: ✉ md_linawati @ unud .ac.id
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fatty liver [4, 5], while green apples contain high fiber, fructose, vitamins (C, E), minerals (potassium, magnesium), triterpenoids, polyphenols (tannins, flavanols, flavonols). The content of phenolics and flavonoids has been widely distributed in plants, and shown antioxidant effects, capture of free radicals, anti-inflammatory and anti-carcinogenic effects. Epidemiological studies showed that apple consumption can lower the risk of some cancers, cardiovascular diseases, asthma, diabetes, fat oxidation, and the progression of atherosclerosis [6, 7]. This study aimed to determine the potential combination of pare and green apple extract in preventing atherosclerosis plaque through the decrease of IL-17 and fatty liver in high-fat diet mice. This study would have benefits to prove the potential effects of pare and apple extracts combination on the pathogenesis of atherosclerosis through the decrease of IL-17 and fatty liver.

MATERIALS AND METHODS

This research was an experimental research with a posttest only control group design, in which there were 7 research groups, each consisting of four Balb/C male mice.

Sample

The study population was mice strain Balb/c (*Mus musculus*) population, 5 weeks old, weighing 20-25 grams, male, healthy with no physical defects. 28 mice were taken randomly to be grouped into 7 study groups.

Preparation of Pare Ethanol Extract (Pa) and Apple Ethanol Extract (Ap)

Pare and green apples in fresh condition were split, removed the seeds, dried in oven in 60^0C for 20 hrs, smashed with a blender, then weighed 100 grams for each. Pare and green apple powder was added to 300 ml of ethanol and stirred with a magnetic stirrer for 1 hour at room temperature, then filtered with Whatman paper to obtain the filtrate 1. The obtained extracts were extracted to obtain filtrate 2. Filtrate 1 and filtrate 2 were mixed, and then evaporated with a rotary evaporator. The dried extracts of pare and apples were then placed in the bottle separately. In the time of using, they would be diluted with the addition of a solution of glycerin according to the calculated amount of the required dose. In this study, 100 mg/kgbw of each extract was used.

Research Procedures

Group 1 (K0) included mice with normal diet (ND) as a negative control; Group 2 (K1) included obese (high-fat diet / HFD) mice as a positive control; Group 3 (P1) got HFD and pare extract (Pa) 100 mg / kgbw; Group 4 (P2) got HFD and green apple extract (Ap) 100 mg / kgbw; Group 5 (P3) got HFD and apple-pare combination extract (APa) 100 mg / kgbw; Group 6 (P4) got HFD and simvastatin preparation (Siv) 10 mg / kgbw; While group 7 (P5) got HFD, APa 100 mg / kgbw and siv 10 mg / kgbw. After getting HFD for 2 weeks, on the 15th day, they were given Pa, Ap, Siv for 2 weeks. Then, on the 29th day, all groups of the study were terminated. The heart organ was processed according to the ELISA procedure (Biolegend) IL-17 examination, whereas the liver was prepared histopathologically by Hematoxylin Eosin (H-E) to check the fatty liver. The data obtained were then analyzed by *One Way Anova*.

Measuring IL-17 concentration by ELISA (Enzyme Linked Immunoassay) method

A. The cardiac tissue was prepared.

1. The mice were terminated according to the euthanasia principle in animal model. (*Ethical Clearance No:* 659 / UN. 14 . 2 / KEP / 2016)
2. The lung tissue size ± 100 µg was taken, and inserted into Eppendorf store at -800C.
3. The lung tissue was destroyed with sterile mortar over dry ice.
4. The cracked tissue was inserted into the Eppendorf tube, and then 10 µl protease inhibitor soaked in water temperature of 40C was added.
5. Homogenization was done at low speed for 20 seconds, and the temperature was kept cold.
6. The sample was moved into a 2 ml micro centrifuge tube, centrifuge at 14.000xg for 15 minutes at 40C.
7. The supernatant was taken, placed on some tubes (aliquot), the ELISA was done according to the kit protocol.

B. ELISA Cardiac tissue sample to measure IL-17 concentration

1. The Standard was prepared.
2. 100 µl standard diluent buffer was entered at wells prepared for the standard.
3. 100 µl standard, control and sample (dilution sample > 1:10) were entered, and then shaken.
4. The well was covered and incubated for 2 hours at room temperature.
5. The liquids 4x were disposed.
6. 100 µl streptavidin-conjugated HRP was entered, and was shaken.
7. The obtained liquid was incubated for 1 hour at room temperature.
8. The liquid was disposed and 4x was washed.
9. 100 µl streptavidin-HRP was incorporated and shaken.

10. Then, it was incubated for 30 minutes at room temperature.
11. 4x was washed.
12. 100 µl Chromogenic was entered on each well, and the color changed to blue.
13. Then, it was incubated for 30 minutes, in room temperature, and darkness.
14. 100 µl stop solution was entered, and the color was changed from blue to yellow.
15. The wavelength was read with ELISA 450 nm wavelength reader, within a maximum of 2 hours after the addition of stop solution.
16. OD (Optical Density) results of each standard, control and sample were obtained.
17. The IL-17 concentrations of each control and sample could be determined by a standard curve using logarithms or calculated by the software.

Histopathological Examination of Hepar tissue

1. The liver organ was prepared for paraffin block making.
2. The paraffin blocks were cut with a microtome with a size of 0.3-0.5 µm and then attached to the glass object.
3. Dehydration was done with xylol 2 x 5 minutes.
4. Rehydration was done with absolute ethanol 2 x 5 minutes followed by washing PBS 1x for 2 x 5 minutes, then it was shaken.
5. Then, it was dried, followed by Hematoxillin-Eosin, and then it was soaked for 5 minutes. Then, it was washed with running water.
6. Dehydration was done with absolute ethanol 2 for 5 minutes.
7. Clearing was carried out with Xylol 2 x 5 minutes.
8. The cover was mounted with a cover slip.
9. Then it was viewed under light microscope Olympus CX3100 with Optilab camera. Microscopic observation was performed to look for changes in the histopathologic features based on fatty liver (steatosis), inflammation of liver cells (inflammation), and degeneration of liver epithelial cells (ballooning). The scoring was based on NASH scoring.

Data Analysis

Data concentration of IL-17 and fatty liver were tabulated, then the homogeneity and normality were analyzed, if it were fulfilled, then it would be continued by One Way Anova ($p < 0.05$), in order to analyze the differences in means between the group of treatment, and test the interaction between each effect. Finally, it was tested by LSD (Least Significant Difference) test so it was known which groups differed significantly.

RESULTS

The Secretion of IL-17 in Mice given High Fat Diets (HFD).

Interleukin 17 concentrations in 7 groups of study can be seen in Table 1 which shows the mean concentration of IL-17 in group K0 (23,772 pg / mL); K1 (138,768 pg / mL); P1 (55,552 pg / mL); P2 (86,458 pg / mL); P3 (49.19 pg / ml); P4 (69,794 pg / mL); P5 (85.416 pg / mL). This showed the highest average concentration of IL-17 in the high-fat diet group (HFD) and the lowest was in the negative control group (K0) which received normal diet (ND). The concentrations of IL-17 in the group receiving HFD and the pare apple extract (APa) were the closest to the K0 group compared to the other groups.

Table 1. Interleukin 17 secretion in the group of study

No	Sample Code	Absorbance	Concentration (pg/mL)	Mean
1	K0	0.095	27.48	23.772
2	K0	0.095	27.48	
3	K0	0.085	23.36	
4	K0	0.078	20.27	
5	K0	0.078	20.27	
6	K1	0.14	137.5	138.768
7	K1	0.136	125	
8	K1	0.144	153.17	
9	K1	0.144	153.17	
10	K1	0.136	125	
11	P1	0.119	55.6	55.552
12	P1	0.107	35.98	
13	P1	0.113	40.34	
14	P1	0.126	72.92	
15	P1	0.126	72.92	
16	P2	0.13	93.75	86.458
17	P2	0.129	88.54	
18	P2	0.129	88.54	
19	P2	0.127	78.13	
20	P2	0.128	83.33	
21	P3	0.112	43.78	49.19
22	P3	0.112	43.78	
23	P3	0.114	48.99	
24	P3	0.119	54.7	
25	P3	0.119	54.7	
26	P4	0.125	67.71	69.794
27	P4	0.127	78.13	
28	P4	0.126	72.92	
29	P4	0.125	67.71	
30	P4	0.124	62.5	
31	P5	0.128	83.33	85.416
32	P5	0.13	93.75	
33	P5	0.128	83.33	
34	P5	0.129	88.54	
35	P5	0.127	78.13	

Focal Aggregation of Liver Inflammation in Mice given High Fat Diet (HFD)

In this study, no fatty liver was found. However, in the seventh group of study, there was an inflammatory aggregate. Figure 1, shows the inflammatory aggregate in K0, K1, P1, P2, P3, P4 and P5 groups. Inflammatory aggregates on liver tissue stained with hematoxylin-Eosin was marked with a circle sign.

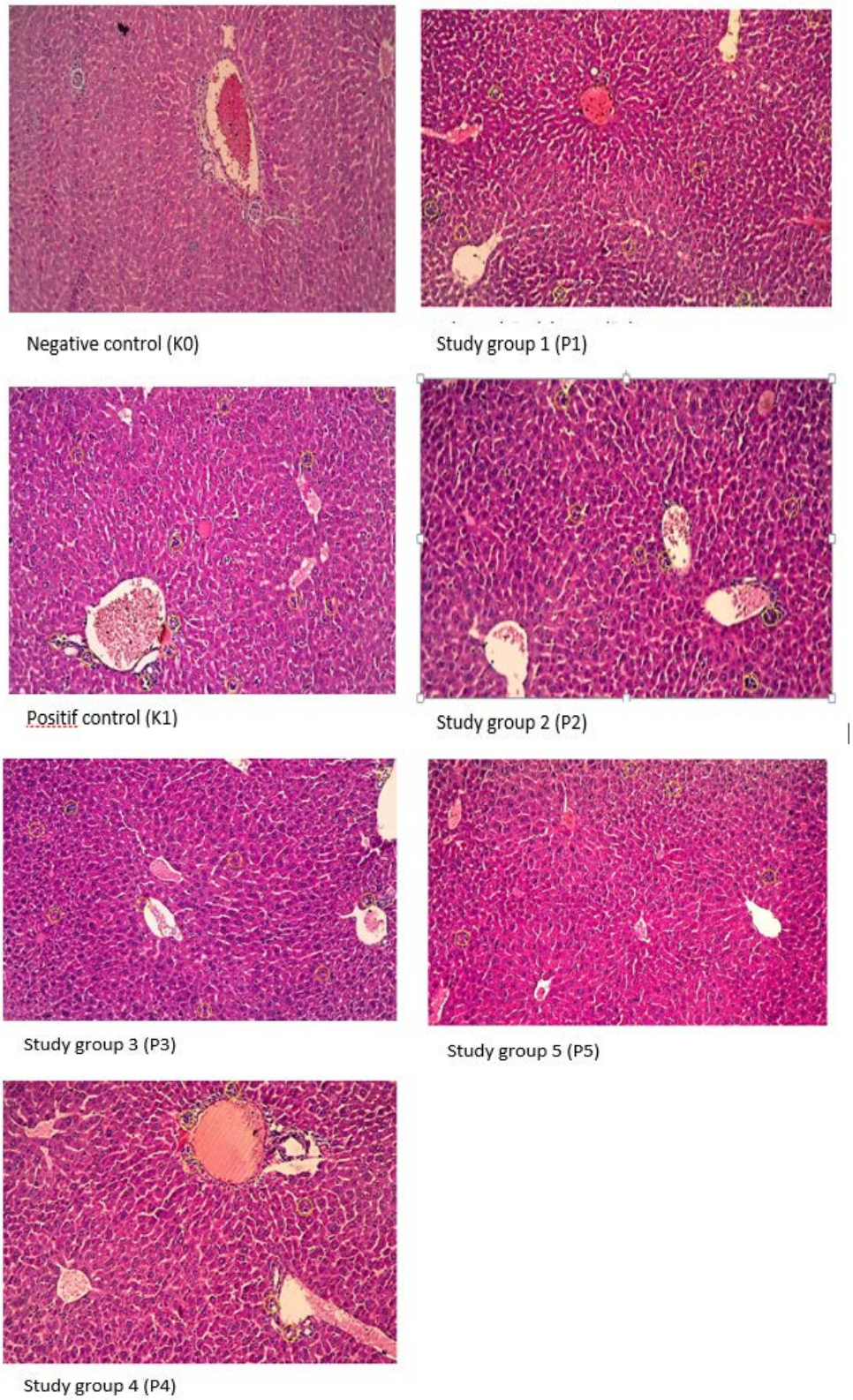

Fig. 1. Focus of Aggregate Inflammation on the Liver Tissues in Study Groups (HE, 100x)

The average of Inflammatory Aggregate (focus area) in group K0 (1,6); K1 (13,2); P1 (11.6); P2 (7,6); P3 (10); P4 (6) and P5 (4), can be seen in table 2. So the highest focus of inflammation was found in group K1, whereas the lowest after group K0 was group P5.

Table 2. Mean of focal Agregate Inflamation

No	Groups	Inflamation Agregate	Mean
1	K0	1	1.6
2	K0	2	
3	K0	2	
4	K0	1	
5	K0	2	
6	K1	14	13.2
7	K1	13	
8	K1	14	
9	K1	12	
10	K1	13	
11	P1	12	11.6
12	P1	11	
13	P1	12	
14	P1	12	
15	P1	11	
16	P2	8	7.6
17	P2	8	
18	P2	7	
19	P2	8	
20	P2	7	
21	P3	10	10
22	P3	9	
23	P3	11	
24	P3	10	
25	P3	10	
26	P4	6	6
27	P4	5	
28	P4	7	
29	P4	6	
30	P4	6	
31	P5	4	4
32	P5	3	
33	P5	4	
34	P5	4	
35	P5	5	

DISCUSSION

The analysis of IL-17 concentration data and focal aggregate inflammation can be seen in table 2. From IL-17 data analysis, it was found that IL-17 concentration was the highest in K1 group who only got high fat diet (HFD). This was in accordance with the Zaraean study [2] who declared that the HFD that causes obesity would induce atherosclerosis. Where in the serum of obese individuals, there was found increased IL-17. The concentrations of IL-17 in the seven groups of the study were significantly different ($p < 0.05$). The administration of apple extract, pare or mixture of Apples and Pare (APa) significantly decreased the concentration of IL-17 compared to K1 group. The concentration of IL-17 was the closest to the concentration of the K0 group as a negative control that only got the normal diet (ND), and P3 group that got HFD and APA extract 20 mg / 20 grbb, with IL-17 concentration of 49.19 pg / mL. This showed the active substance content in the combined extract of apples and pare which works in synergy. The lower IL-17 concentration was in accordance with a research done by Koutsos [6] who stated that the content of polyphenols in apples has been proven to lower serum cholesterol and liver after hyper cholesterol diet. In addition, Krawinkel [7] showed that pare extract has been proven to decrease the obesity induced by HFD and hyperlipidemia. So, both apple and pare extracts induced cholesterol depletion, which would reduce the incidence of obesity and IL-17 concentrations. Previous studies showed a significant decrease in triglyceride, total cholesterol, CRP and arteriosclerosis lesions and increased LDL cholesterol in rabbits after being given apple juice with a dose of 10 ml of apple juice / day for 60 days [8]. The data analysis of the focal aggregate inflammation on liver tissue showed the highest focal aggregate inflammation in the K1 group which received HFD only. The lowest inflammatory aggregate concentration after the K0 group was found in the P5 group receiving HFD, simvastatin 0.04 mg / 20 grbb and APA extract 20 mg / 20 grbb. This showed that APA extract can be used as adjuvant therapy for hypercholesterolemia in addition to standard simvastatin therapy. APA extract works synergistically with simvastatin in reducing the inflammatory aggregate focus on the liver. The focus of inflammatory aggregates from the liver is a pathological picture that initiates the onset of fatty liver. With the potential to decrease the inflammatory aggregate focus, combined APA extracts could potentially prevent fatty liver in experimental animals which was given HFD. This was in line with Zhang's study (2013) where the pare supplementation reduced the accumulation of fatty liver and prevented fatty liver (steatosis) in mice given a HFD [9]. However, the high fiber content, fructose, vitamin (C, E), minerals (potassium, magnesium), triterpenoids, polyphenols (tannins, flavanols, flavonols) in apples worked synergistically with pare and simvastatin preparations, so the inflammatory aggregate focus was the lowest in Group P5. In addition to polyphenol content, pectin in apples lowered cholesterol by inhibiting cholesterol absorption, affecting micelle formation and transiting time. The synergistic effect between pectin and polyphenols in apples could reduce fat [10]. Polyphenols are potential substances against cancers and cardiovascular, metabolic, and neurodegenerative diseases through their abilities of anti oxidation and anti mutation. The metabolism of polyphenols can neutralize free radicals by donating an electron or hydrogen atom to suppress the generation of free radicals, or deactivate the active species

and precursors of the free radicals. Polyphenols, as metal chelates, and chelate metal transition such as Fe^{2+} directly reduced the rate of Fenton reaction, thus preventing oxidation caused by highly reactive hydroxyl radicals (•OH) [11]. Polyphenols have also antithrombotic effects, which could be attributed to reduced susceptibility to platelet activation and aggregation, reduced synthesis of prothrombotic mediators (eicosanoid synthesis), and decreased gene expression of tissue factor. Resveratrol has been shown to inhibit platelet aggregation induced by collagen, ADP, and thrombin in a concentration-dependent manner. Mattiello et al. compared the effect of pomegranate juice and that of the polyphenol-rich extract from pomegranate fruit on platelet aggregation, calcium mobilization, thromboxane A2 production, and hydrogen peroxide formation induced by collagen and arachidonic acid. Both the pomegranate juice and the extract reduced all platelet responses, with the latter showing a stronger effect [12] As having good anti oxidative abilities of polyphenols, they may play important roles and interact with some cell receptors and intracellular signaling and/or gene expression regulation during atherosclerotic progressions. Pare / bitter melon supplementation has proven significantly decreased body weight gain by increasing the hepatic and muscle mitochondrial carnitine palmitoyltransferase-I (CPT-1) and Acyl-CoA dehydrogenase enzyme. Carnitine palmitoyltransferase (CPT) system is the predominant system for transporting the fatty acid to mitochondrial matrix. The plant extracts may modulate fat metabolizing kinases such as AMPKs, genes, and nuclear factors like PPARs, LXRs, and PGC-1α, in liver and skeletal muscle and affected adipocyte differentiation, while several review papers suggested the antidiabetic mechanism [13]. Apples which contain polyphenol (APs) have had anti-atherogenic and anti-obesity effects of APs, which have been related to the decreased plasma TC and TG levels. APs treatment elevated the plasma levels of leptin, which plays an important role in the regulation of body weight and energy balance. It has also been reported that the deficiency of either leptin or its receptor has resulted in marked increases in both plasma cholesterol and triglyceride concentrations and increased lesion formation. Leptin-deficient mice have the potential for hypertriglyceridemia and hypercholesterolemia, so the risk of atherosclerosis could be increased [14].

CONCLUSION

1. APa combined extract could decrease the concentration of IL-17 heart tissue of mice given a high-fat diet (HFD) significantly (P <0.05)
2. APa combined extract could decrease the aggregate inflammatory in liver tissue of mice given a high-fat diet (HFD) significantly (P <0.05).

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